Antibody responses to cancer antigens identify patients with a poor prognosis among HPV-positive and HPV-negative head and neck squamous cell carcinoma patients

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Abstract: Purpose: The identification of high-risk patients within Human Papillomavirus (HPV) positive and negative head and neck squamous cell carcinoma patients is needed for improved treatment and surveillance strategies. In this study, we set out to discover Antibody responses (AR) with prognostic impact in head and neck squamous cell carcinoma (HNSCC) stratified by HPV-status. Experimental Design: A fluorescent bead-based multiplex serology assay to 29 cancer antigens (16 cancer-testis antigens, 5 cancer-retina antigens, 8 oncogenes) and 29 HPV-antigens was performed in samples of 362 HNSCC patients from five independent cohorts (153 HPV-positive, 209 HPV-negative). A multivariable cox proportional hazard model with bootstrapping (M=1000) was used for validation of prognostic antibody responses. Results: AR to any of the cancer antigens were found in 257/362 patients (71%). In HPV-negative patients, antibody responses to to c-myc, MAGE-A1, -A4 and Rhodopsin E2 (combined as ARhigh risk) were significantly associated with shorter overall survival. In HPV-positive patients antibody responses to IMP-1 were discovered as a negative prognostic factor. ARhigh risk (HR=1.76) and antibody responses to IMP-1 (HR=3.28) were confirmed as independent markers for a poor prognosis in a multivariable Cox proportional hazard model with bootstrapping (M=1000). Conclusion: We identified AR to cancer antigens that associate with a dismal prognosis in HNSCC patients beyond HPV-positive-status. ARhigh risk may be used to detect HPV-negative patients with an extraordinarily bad prognosis. Most importantly, AR to IMP-1 may serve as a marker for a subgroup of HPV-positive patients that present with a poor prognosis similar to that in HPV-negative patients.

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Running Title: Prognostic impact of AR to cancer antigens in HNSCC

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Antibody responses to cancer antigens identify patients with a poor prognosis among HPV-positive and HPV-negative head and neck squamous cell carcinoma patients

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Statement of translational relevance:

Human Papillomavirus (HPV) driven head and neck squamous cell carcinoma (HNSCC) is characterized by a much better prognosis than HPV-negative HNSCC. Recent studies focus on de-escalation of treatment. However, among HPV-positive patients, a subgroup with a dismal prognosis exists. In order to prevent harm from treatment de-escalation for such patients, biomarkers for high-risk patient identification among HPV-positive patients are needed.

We analyzed antibody responses to non-viral cancer antigens for HPV-positive and HPV-negative patients (n=362) in five independent cohorts treated at large European cancer centers. Antibodies to IMP-1 were associated with reduced overall survival in HPV-positive patients, and antibody responses to c-myc, MAGE-A1, MAGE-A4, and Rhodopsin E2 (combined as $AR_{high\ risk}$) in HPV-negative patients respectively. Our findings were validated in a multivariable cox proportional hazard model with bootstrapping (M=1000). In HPV-positive and negative head and neck squamous cell carcinoma, antibody responses to cancer antigens may be used to identify high-risk patients.

Abstract

Purpose: The identification of high-risk patients within Human Papillomavirus (HPV) positive and negative head and neck squamous cell carcinoma patients is needed for improved treatment and surveillance strategies. In this study, we set out to discover Antibody responses (AR) with prognostic impact in head and neck squamous cell carcinoma (HNSCC) stratified by HPV-status.
Experimental Design: A fluorescent bead-based multiplex serology assay to 29 cancer antigens (16 cancer-testis antigens, 5 cancer-retina antigens, 8 oncogenes) and 29 HPV-antigens was performed in samples of 362 HNSCC patients from five independent cohorts (153 HPV-positive, 209 HPV-negative). A multivariable cox proportional hazard model with bootstrapping (M=1000) was used for validation of prognostic antibody responses.
Results: AR to any of the cancer antigens were found in 257/362 patients (71%). In HPV-negative patients, antibody responses to to c-myc, MAGE-A1, -A4 and Rhodopsin E2 (combined as $AR_{high\ risk}$) were significantly associated with shorter overall survival. In HPV-positive patients antibody responses to IMP-1 were discovered as a negative prognostic factor. $AR_{high\ risk}$ (HR=1.76) and antibody responses to IMP-1 (HR=3.28) were confirmed as independent markers for a poor prognosis in a multivariable Cox proportional hazard model with bootstrapping (M=1000).
Conclusion: We identified AR to cancer antigens that associate with a dismal prognosis in HNSCC patients beyond HPV-positive-status. $AR_{high\ risk}$ may be used to detect HPV-negative patients with an extraordinarily bad prognosis. Most importantly, AR to IMP-1 may serve as a marker for a subgroup of HPV-positive patients that present with a poor prognosis similar to that in HPV-negative patients.

Introduction

Globally, head and neck squamous cell carcinoma (HNSCC) is diagnosed in almost 900,000 cases annually resulting in approximately 450,000 cancer deaths per year (1). Human papillomavirus (HPV) positive oropharyngeal squamous cell carcinoma (OPSCC)(2) and head and neck cancer of unknown primary (CUP)(3) have been recognized as distinct entities of HNSCC causally associated with HPV. Clinically, a significant prognostic advantage for HPV-positive OPSCC has been determined in numerous studies for different primary treatment strategies (4, 5). However, five year overall survival rates of 70-80% indicate the existence of a subgroup with a dismal prognosis within HPV-positive patients. To date, there is no robust biomarker to detect such patients.
Cancer-antigens are immunogenic proteins or peptides that can be recognized by the immune system. Shared cancer antigens include germ-line antigens that are exclusively expressed in tumor tissue such as cancer-testis antigens (6, 7) or cancer-retina antigens (8), oncogenes overexpressed in cancer tissue such as p53 (9) and foreign antigens such as viral antigens (10). Whereas antibody responses (AR) to viral antigens can be used to identify HPV-positive patients (3, 11-14), AR to shared, non-viral antigens may play an important role as prognostic markers in both HPV-positive and HPV-negative HNSCC (15, 16).
The aim of this study was to define the potential prognostic impact of such AR to cancer antigens in HNSCC patients stratified by HPV-status.

Methods

The study was performed in accordance with the EQUATOR Network CONSORT Guidelines for prognostic studies, namely the *Reporting Recommendations for Tumor Marker Prognostic Studies* (REMARK Guidelines) (17).

Patients

In this study, 362 patients with curative treatment for histologically diagnosed head and neck squamous cell carcinoma (HNSCC), an available serum or plasma sample taken prior to the initiation of treatment and written informed consent according to the Helsinki Declaration II were selected. Patients were treated per institutional guidelines at five large head and neck cancer centers, namely University Medical Centers Ulm, Heidelberg Leipzig (Germany), Padua (Italy) and St. Gallen (Switzerland) (Figure S1). The AJCC cancer staging manual version 7 was used for classification of TNM and disease stage.

Human papilloma virus status

HPV status was determined at each center according to institutional standards. For n=294 patients, a multiplex HPV-DNA PCR (GP5+/GP6+ primers followed by Sanger sequencing for HPV typing as previously described (18)) and p16 immunohistochemistry (n=254) was performed. For n=146 patients, HPV E6*I mRNA status(19, 20) was available. Molecular HPV-status was considered positive if two of the following three parameters were positive: HPV-DNA of known high-risk types, HPV-16 E6*I RNA, p16 immunohistochemistry. All other combinations were considered HPV-negative. Molecular HPV-status showed a significant correlation with the results of HPV-serology (Pearson correlation coefficient = 0.775, $p<0.001$). Thus, for patients lacking data for determination of the molecular HPV status, primarily non-oropharyngeal cancers, results from HPV-serology to high-risk types were used as a surrogate parameter resulting in a combined marker HPV (mol/ser).

Material

Serum or plasma samples were prospectively collected prior to treatment initiation, aliquoted and stored at -20°C until use. Prospective sample collection was in accordance with local ethics committee approvals .

Cancer-antigen serology

Full-length proteins of selected cancer antigens were generated for multiplex serology as previously described (21-24). The cancer antigen panel is shown in Table 1.
In brief, genes encoding for 16 cancer-testis antigens, 5 cancer-retina antigens, 8 oncogenes, 29 HPV-antigens (from 8 high-risk HPV types) and two control antigens (JC and BK virus major capsid protein VP1) were cloned into the pGEX4T3 tag vector for expression in *E. coli* BL21 as fusion proteins with N-terminal glutathione-S-transferase (GST) and a small C-terminal tagging epitope (tag) as previously described (23, 25). GST-tag fusion protein without insert was used to determine serological background. Anti-GST (GEHealthcare, Munich), anti-tag and anti-mouse HRP secondary antibodies (Dianova) were used to confirm full-length protein expression and protein integrity.
Multiplex serology was performed as previously described (21-23, 25). For each antigen and bead set, 2500 glutathione-casein coated beads per sample were used and sera or plasma were measured at 1:1000 dilutions. Reporter fluorescence of the beads was determined with the Bio-Plex analyzer (BioRad) and expressed as MFI of at least 100 beads per set per well. Antigen-specific reactivity was calculated as the difference between antigen-MFI, GST-tag-MFI and a blank. This value was used for further analyses. Cut-offs were determined graphically for non-viral antigens based on visual inspection of percentile plots (26). For viral antigens, cut-offs were available from previous studies (12).

Statistics

For statistical analysis, the SAMPL guidelines were respected (27). *IBM SPSS statistics version 25.0* was used for statistical analysis unless indicated otherwise.
Survival data were available for 360/362 patients. Overall survival (OS) was defined as the time interval from diagnosis until death. Disease-specific survival (DSS) was defined as the time interval from diagnosis until cancer-related death. Non-cancer related deaths were not counted as events for DSS. Survival analyses were performed and graphed with SPSS using the Kaplan-Meier method. Analyses with <5 patients in one of the groups analyzed were excluded from OS analysis. Groups were compared by log-rank-test. P-values <0.05 were considered significant, but corrections for multiple testing were performed to reduce statistical errors. Corrections for multiple testing were done using *Prism version 7.0c* (*GraphPad Software, Inc*) with a false discovery rate (FDR) approach for each hypothesis using the two-stage step up method of Benjamini, Krieger and Yekutieli (28). Given the exploratory nature of the study, a FDR of up to 15% was tolerated.
Multivariable Cox proportional-hazards models were used to calculate hazard ratios (HR) and 95% confidence intervals (CI) using R. We applied a bootstrap approach (M=1000) for variable selection (29). This method uses a nested selection procedure over all variable subsets and model comparison via Akaike information criterion to determine the most relevant variables, i.e. only the most frequent variables (>=70%) from all bootstrap replications. The following known prognostic markers were included in the multivariable analysis: T-status (T1-3, T4, CUP), N-status (N0, N+), stage (I, II, III, IV), HPV-status (mol/ser), smoking status (yes, no), primary treatment modality (surgical, non-surgical) and the primary site (Oropharynx, CUP, non-Oropharynx). In addition, all experimental markers that were significantly associated with survival in the whole cohort or stratified by HPV-status were integrated into the model.

Results

Patient characteristics of the cohort of 362 patients with HNSCC are presented in Table 2. The majority of patients had an oropharyngeal tumor, explaining the high rate of HPV-positive patients (42.3%). A more detailed description of the non-oropharyngeal tumor patients is presented in supplementary Table S1.

Among the 362 patients, 257 (71%) were seropositive for any of the 29 auto-antigens tested. Within the cohort 360 patients were evaluable for OS and 119 deaths occurred during the follow-up interval. Cause of death was available in 106/119 deaths (89%). Among those 106 deaths 70 (66%) were cancer-related.

To identify a prognostic impact associated with the presence of AR to certain antigens, overall survival (OS) was analyzed using the Kaplan-Meier method for each of the autoantigens. Patient groups containing <5 patients in one of the groups to be compared were excluded from the analysis. As shown in Figure S1, ≥ 2 AR, AR to IMP-1, MAGE-A1, -A3, -A4, -A9, p53 Rhodopsin E2 and SSX-2 were associated with significantly shorter OS after correction for multiple testing with a FDR of 15%.

The prognostic impact of HPV-status is well-known and can be considered the most important prognostic factor for HNSCC (4, 5, 13). In this cohort, the molecular HPV-status, as well as the serologic HPV-status (HPV-16 E6 antibodies, antibodies to high risk HPV-types) and the combined surrogate marker HPV (mol/ser) resulted in consistent survival differences compared to the respective HPV-negative group (supplementary Figure S2). AR patterns and prevalences differed between HPV-positive and HPV-negative patients (not shown). Thus, OS analyses were then performed stratified by HPV-status (HPV mol/ser). Interestingly, ≥ 2AR to autoantigens, AR to c-myc, MAGE-A1, -A4 and Rhodopsin E2 were significantly associated with shorter OS in HPV-negative patients, but not in HPV-positive patients (Figure S3). On the other hand, AR to IMP-1 were significantly associated with shorter OS in HPV-positive patients (p>0.001), but not in HPV-negative patients (p=0.150) (Figure 1). Moreover, HPV-positive patients with AR to IMP-1 did not have a significantly different prognosis compared to HPV-negative patients (p=0.5). The results were corrected for multiple testing with a FDR of 15%. Results showed the lowest q-value for c-myc in HPV-negative patients (q<0.001) and for IMP-1 in HPV-positive patients (q<0.002).

AR to any antigen with prognostic impact in HPV-negative patients, namely c-myc, MAGE-A1, MAGE-A4 or Rhodopsin E2, were subsequently summarized under a new variable $AR_{high\ risk}$ (n=83). Presence of $AR_{high\ risk}$ was associated with significantly shorter survival in HPV-negative patients compared to patients lacking $AR_{high\ risk}$ or HPV-positive patients (p<0.001; Figure 2). In HPV-positive patients, no statistical survival difference of patients with $AR_{high\ risk}$ compared to those without $AR_{high\ risk}$ (p=0.850) was found. The results for DSS were not substantially different (not shown).

In order to address bias from other known prognostic markers, a multivariable Cox proportional hazard model was used. In addition to the known prognostic markers described in the methods section, all AR with a potential prognostic impact based on the OS analysis in the different patient groups (all, HPV-positive, HPV-negative) were tested, namely MAGE-A3 AR (AR-, AR+), MAGE-A9 AR (AR-, AR+), p53 AR (AR-, AR+), SSX2 AR (AR-, AR+), $AR_{high\ risk}$, IMP1 AR (AR-, AR+) and the number of AR (0-1, ≧2). In the final model, advanced T-category, $AR_{high\ risk}$ and AR to IMP-1 were associated with a significantly increased hazard ratio (HR), whereas HPV-positive status was associated with a significantly reduced HR (Cox proportional hazard model; Wald: p=6e-11). HR values for the four variables in the final model with the respective 95% confidence intervals, p-values and the number of positive bootstraps are listed in Table 3.

Discussion

We were able to show a significant prognostic impact for shorter OS associated with several AR to cancer antigens, both agnostic of and stratified by HPV-status. The multivariable Cox proportional hazard models confirmed $AR_{high\ risk}$ and IMP-1 among T-category and HPV-status ($HPV_{mol/ser}$) as independent markers of a poor prognosis. Instead of arbitrarily dividing the cohort from the five centers into just one test and one validation cohort, we selected a bootstrap approach (M=1000) for variable selection (29). The benefit of this method is a nested selection procedure over all variable subsets. All models were compared via Akaike information criterion to determine the most relevant variables. Only the most frequent variables (>=70%) from all bootstrap replications were selected for the final model. $AR_{high\ risk}$ and IMP-1 were stably presented variables in the 1000 models tested.
Thus, we have identified a serological marker each to detect patients at higher risk of death for HPV-negative patients ($AR_{high\ risk}$) and for HPV-positive patients (IMP-1).
IMP-1 (Insulin-like growth factor 2 mRNA-binding protein 1; IGF2BP1) is a mRNA binding protein that functions as a transcription factor and also belongs to the cancer-testis antigen family (30). By binding to mRNA it forms a protein mRNA complex which stabilizes the target RNA and enhances translation of protein e.g. of cmyc (31) or KRAS (32). It is considered to promote cell proliferation and tumorigenesis (31, 33, 34) and has been associated with a poor prognosis in neuroblastoma (35). Humoral immune responses to IMP-1 have previously been described in ovarian cancer (30).
Within the group of HPV-positive patients, a biomarker to identify those HPV-positive patients who have a prognosis comparable to HPV-negative patients is lacking. Smoking status has been reported as a prognostic marker for HPV-positive patients (4), but was not associated with a poor prognosis in our multivariable model. This may be due to different smoking habits in Germany. German HPV-positive patients tend to have a smoking history above 10 pack years in the median (36). In view of efforts do de-escalate treatment for HPV-positive patients, it is highly important to detect high-risk patients.
Recently, two treatment de-escalation trials for HPV-positive patients were published, both only using p16 for determination of HPV-status (37, 38). In contrast to the expected outcome, both trials failed to prove that cetuximab is less toxic than high-dose cisplatin. Instead exchanging cisplatin for cetuximab had a detrimental impact on OS.
Alarmingly, in the clinical routine in the USA, some physicians are not recommending adjuvant treatment to surgically treated HPV-positive patients (39). To protect HPV-positive patients with a bad outcome from harm, the subgroup of patients with a poor prognosis needs to be identified, if a de-escalation of treatment for HPV-positive patients should be established in the future.
Several other studies have associated AR to certain cancer antigens with detrimental or beneficial prognosis (22, 24, 40). In most studies and for most antigens, a negative prognostic impact- consistent with our results- was found. These findings indicate that humoral immunity to cancer antigens may be a poor surrogate marker for active cancer immunity, but rather an indirect measure of antigen expression as shown previously (40-43). At the same time, these patients may be candidates for antigen-specific immunotherapy which may improve the detrimental outcome. Cancer antigen serology may therefore identify patients who are at high risk of death, but who may benefit from immunotherapy (44).
There are some limitations to our study: Patients were not treated within a prospective clinical trial with a defined treatment regimen. However, patients were treated at five large European cancer centers in line with international treatment guidelines. Due to the number of hypotheses tested, corrections for multiple testing were needed and performed. Some of the prognostic groups compared were rather small due to the diversity of antibody response patterns. However, the results for $AR_{high\ risk}$ and IMP-1 remained stable up to a FDR of 1%.

The multivariable Cox proportional hazard model with 1000 bootstraps also confirmed these two factors among T-category and HPV-status to be significantly correlated with OS. The prognostic impact of serologic antibodies detected at baseline is somewhat controversial (3, 22, 24, 45, 46). Antibody levels and biological activity may change over the course of the disease. As such, cancer-antigen antibodies may represent an interesting biomarker during post-treatment surveillance. However, a prospective validation of these new markers, preferably in a randomized trial with samples taken over the course of treatment and follow-up would be desirable.
In conclusion, our results show that $AR_{high\ risk}$ may be used to identify patients with a dismal prognosis among HPV-negative patients and AR to IMP-1 among HPV-positive patients. AR to IMP-1 is a novel marker to detect HPV-positive patients who have a comparable prognosis to HPV-negative patients. In view of current strategies to de-escalate treatment for HPV-positive patients (39) such patients are at risk and need to be identified.

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Figure Legends:

Figure 1: Overall survival of patients with antibody responses to IMP-1.
Kaplan Meier plots are shown for Human papillomavirus (HPV) positive and HPV-negative patients with or without antibody responses (AR) to IMP-1. HPV+ / AR IMP-1+ patients (mean OS 41.2 months) had a much shorter overall survival than HPV+ / AR IMP-1- patients (mean OS 109.3 months, $p<0.001$). In fact, HPV+ / AR IMP-1+ patients (mean OS 41.2 months) had a similar prognosis to HPV- / IMP-1- patients (mean OS 27.0 months, $p=0.530$) or HPV- / IMP-1+ patients (79.7 months, $p=0.515$).

Figure 2: Overall survival of patients with antibody responses to $AR_{high\ risk}$.
Antibody responses (AR) to c-myc, MAGE-A1, -A4 or Rhodopsin E2 were summarized under the variable $AR_{high\ risk}$. Kaplan Meier plots are shown for Human papillomavirus (HPV) positive and HPV-negative patients with or without antibody responses ($AR_{high\ risk}$).
HPV- / $AR_{high\ risk}$ patients (mean OS 36.5 months) had a significantly shorter OS than HPV-/ no $AR_{high\ risk}$ patients (mean OS 87.3 months, $p<0.001$), HPV+/ no $AR_{high\ risk}$ patients (mean OS 105.5 months, $p<0.001$) or HPV+/ $AR_{high\ risk}$ patients (mean OS 70.3 months, $p<0.001$).

Tables

Table 1: Cancer antigen panel for multiplex serology

Cancer-Testis-Antigens (n=16)		Cancer-Retina Antigens (n=5)		HPV-31 (L1, E6, E7)
MAGE-A1	SpanXa1	Arrestin	HSP 70	HPV-33 (L1, E6, E7)
MAGE-A3	SSX2	Recoverin	Ras	HPV-35 (L1, E6, E7)
MAGE-A4	SSX4	Rhodopsin C	p53	HPV-45 (L1, E6, E7)
MAGE-A9	IMP1 (IGF2BP1)	Rhodopsin N	pRb	HPV-52 (L1, E6, E7)
MAGE-C2	cTAGE 5a	Rhodopsin E2	Survivin	HPV-58 (L1, E6, E7)
CT47	CAMEL	Oncogenes (non-viral) (n=8)	HPV-Antigens (n=29)	Control Antigens (n=2)
GAGE	NY-ESO-1	c-myc	HPV-16 (L1, E6, E7, E1, E2, E4)	BK Virus Protein 1
LAGE	OY-TES-1	cyclin D1	HPV-18 (L1, E6, E7, E1, E2)	JC Virus Protein 1

Table 2: Patient characteristics by Primary Site (Oropharynx, CUP, Non-Oropharynx)

		Primary Site						Total Cohort	
		Oropharynx (54,9%)		CUP (11.8%)		Non-Oropharynx (44,0%)			
		n	%	n	%	n	%	n	%
T	1	28	14,0%	n.a.	n.a.	22	18,0%	50	13,8%
	2	86	43,0%	n.a.	n.a.	32	26,2%	118	32,6%
	3	35	17,5%	n.a.	n.a.	29	23,8%	64	17,7%
	4	51	25,5%	n.a.	n.a.	39	32,0%	90	24,9%
	Missing	0	0,0%	n.a.	n.a.	0	0,0%	43	11,9%
	total	200	100%	40	100%	122	100%	362	100%
N	0	41	20,5%	0	0,0%	61	50,0%	102	28,2%
	1	26	13,0%	15	37,5%	19	15,6%	60	16,6%
	2	125	62,5%	20	50,0%	36	29,5%	181	50,0%
	3	8	4,0%	4	10,0%	6	4,9%	18	5,0%
	Missing	0	0,0%	1	2,5%	0	0,0%	1	0,3%
	Total	200	100%	40	100%	122	100%	362	100%
M	0	200	100,0%	40	100,0%	122	100,0%	362	100,0%
	Total	200	100%	40	100%	122	100%	362	100%
Stage	I	6	3,0%	0	0,0%	14	11,5%	20	5,5%
	II	17	8,5%	0	0,0%	17	13,9%	34	9,4%
	III	31	15,5%	15	37,5%	30	24,6%	76	21,0%
	IVA/B	146	73,0%	24	60,0%	61	50,0%	231	63,8%
	Missing	0	0,0%	1	2,5%	0	0,0%	1	0,3%
	Total	200	100%	40	100%	122	100%	362	100%
HPV status (mol or ser*)	HPV negative	77	38,5%	29	72,5%	102	83,6%	208	57,5%
	HPV positive	123	61,5%	11	27,5%	20	16,4%	154	42,5%
	Missing	0	0,0%	0	0,0%	0	0,0%	0	0,0%
	Total	200	100%	40	100%	122	100%	362	100%
Treatment approach	Surgical	148	74,0%	36	90,0%	100	82,0%	284	78,5%
	Non-surgical	52	26,0%	4	10,0%	20	16,4%	76	21,0%
	Missing	0	0,0%	0	0,0%	2	1,6%	2	0,6%
	Total	200	100%	40	100%	122	100%	362	100%
Sex	Male	151	75,5%	36	90,0%	99	81,1%	286	79,0%
	Female	49	24,5%	4	10,0%	23	18,9%	76	21,0%
	Total	200	100%	40	100%	122	100%	362	100%
Smoking	Non-smoker	50	25,0%	8	20,0%	30	24,6%	88	24,3%
	Smoker	148	74,0%	30	75,0%	89	73,0%	267	73,8%
	Missing	2	1,0%	2	5,0%	3	2,5%	7	1,9%
	Total	200	100%	40	100%	122	100%	362	100%

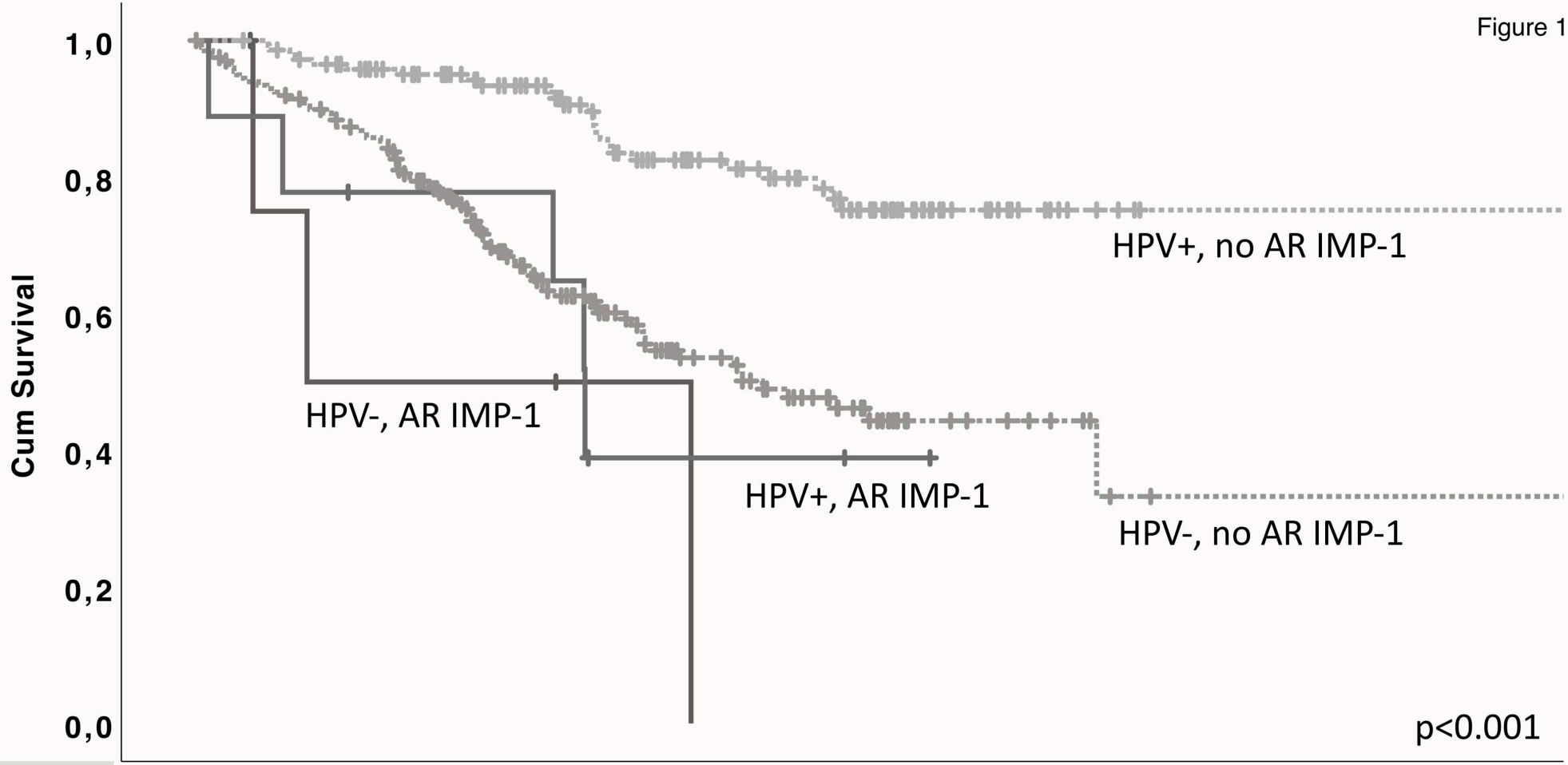
* Molecular HPV status was considered positive if two out of three diagnostic tests (HPV RNA, HPV DNA, p16) were positive. Serology to HPV antigens (compare Table 1) was used whenever molecular status was not available.

Table 3: Final multivariable Cox proporional hazard model based on 1000 bootstraps

		HR	95% CI		p-value	positive bootstrap replicates
T-status	T1-3	1				
	T4	1,750	1,158	2,644	0,008	791
	CUP	2,460	1,420	4,261	0,001	
HPV-status (mol/ser)	HPV-	1			<0,0001	1000
	HPV+	0,340	0,222	0,521		
ARhigh risk	AR-	1			0,007	716
	AR+	1,756	1,167	2,642		
IMP-1 AR	AR-	1			0,002	859
	AR+	3,279	1,562	6,882		

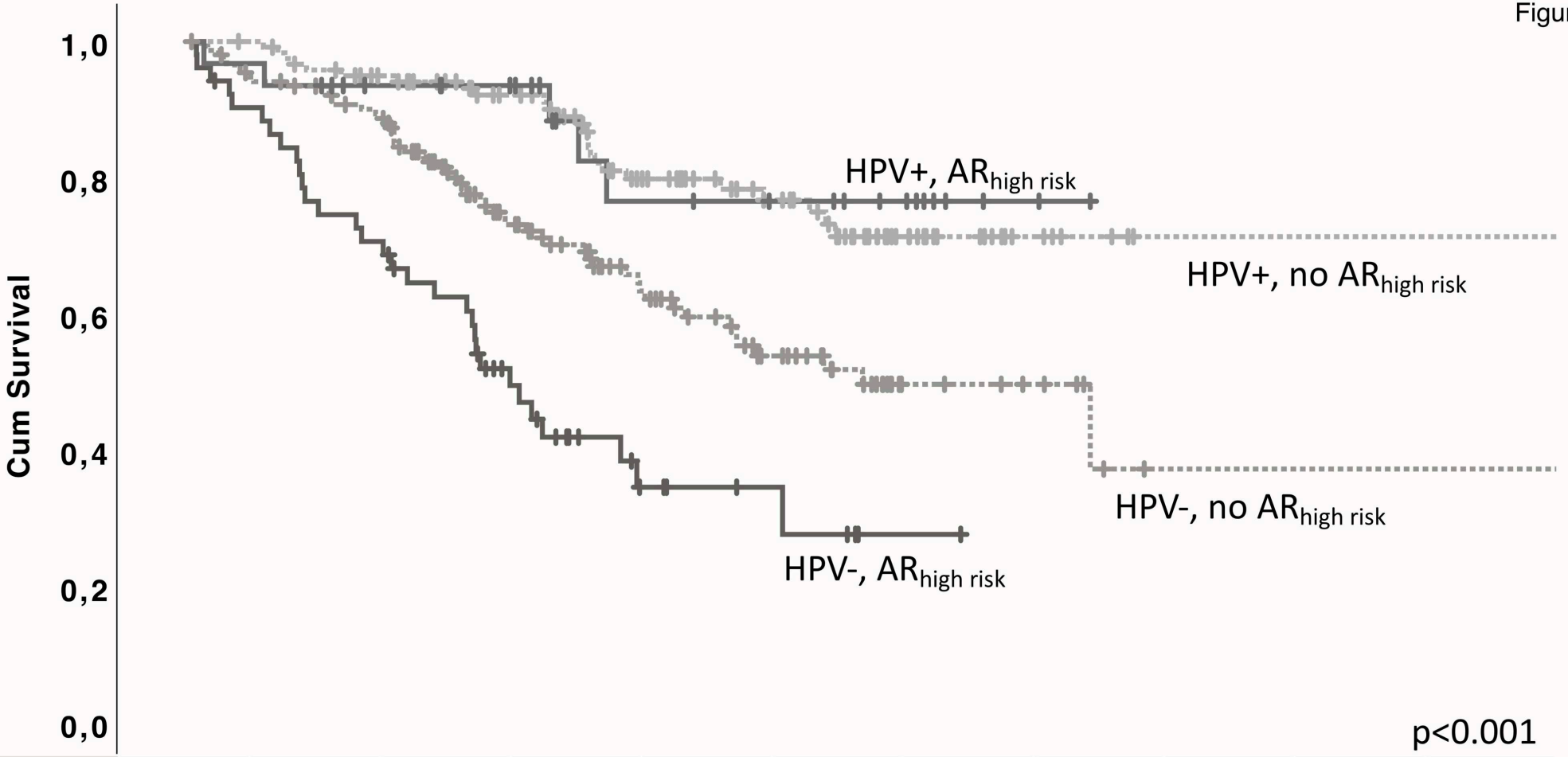
Parameters in the model initially (1000 bootstraps): T-status (T1-3, T4, CUP), N-status (N0, N+), stage (I, II, III, IV), HPV-status (mol/ser), smoking status (yes, no), primary therapy (surgical, non-surgical), Primary site (Oropharynx, CUP, non-Oropharynx), MAGE-A3 AR (AR-, AR+), MAGE-A9 AR (AR-, AR+), p53 AR (AR-, AR+), SSX2 AR (AR-, AR+), AR HPV-negative, IMP1 AR (AR-, AR+), AR# (0-1, ≧2).

Figure 1



Overall Survival (months)	0	12	24	36	48	60	72	84	96	108	120
N at risk HPV-, no AR IMP-1	201	175	130	79	49	28	9	3	1	1	1
N at risk HPV-, AR IMP-1	5	2	2	1	0	0	0	0	0	0	0
N at risk HPV+, no AR IMP-1	145	137	117	91	65	47	17	4	1	1	1
N at risk HPV+, AR IMP-1+	9	7	6	5	2	2	2	2	2	2	2

Figure 2



Overall Survival (months)	0	12	24	36	48	60	72	84	96	108	120
N at risk HPV-, no $AR_{high\ risk}$	155	139	103	68	44	24	9	3	1	1	1
N at risk HPV-, $AR_{high\ risk}$	52	39	30	13	6	4	4	4	4	4	4
N at risk HPV+, no $AR_{high\ risk}$	122	114	99	80	54	39	14	4	1	1	1
N at risk HPV+, $AR_{high\ risk}$	31	29	23	15	12	10	3	3	3	3	3

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